AD_____

GRANT NUMBER DAMD17-96-1-6098

TITLE: Investigations of Functional and Structural Interactions Between c-src and HER2: Involvement in Human Breast Tumor Formation

PRINCIPAL INVESTIGATOR: Allison P. Belsches, Ph.D.
Sarah J. Parsons, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22908

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1997	Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE Investigations of Functional and Structural Interactions Between c-src and HER2: Involvement in Human Breast Cancer Tumor Formation

5. FUNDING NUMBERS
DAMD17-96-1-6098

6. AUTHOR(S)
Allison P. Belches, Ph.D.
Sarah J. Parsons, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, VA 22908

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Studies have shown that functional interaction between members of the human EGF receptor (HER) receptor tyrosine kinase family, and c-Src, a non-receptor tyrosine kinase, may be involved in breast tumor formation. We have demonstrated overexpression of HER1 and c-Src, in a murine fibroblast cell line, C3H10T1/2, results in an EGF-dependent synergistic increase in tumorigenicity, as compared to overexpression of either kinase alone. This increase in tumorigenicity correlates with formation of *in vivo* HER1/c-Src complexes, the appearance of two novel phosphorylation sites on HER1, and enhanced phosphorylation of receptor substrates. HER2 is overexpressed in 20-30% of human breast cancers, and is correlated with poor patient prognosis. Also, elevated levels and/or activity of c-Src have been demonstrated in human breast cancers. We propose HER1 and HER2 interact with c-Src through similar mechanisms to augment cellular growth and tumor progression. To examine potential interactions between HER2 and c-Src, we have studied human breast tumor tissue, and a panel of human breast carcinoma cell lines, for expression of HER family members and c-Src protein. Tumor samples and cell lines were also tested for HER2/c-Src heterocomplexes. In 3 of 13 tumor tissues, and in 3 of 9 human breast tumor cell lines, constitutive *in vivo* HER2/c-Src complexes were seen, suggesting structural and functional interactions between HER2 and c-Src. C3H10T1/2 fibroblast cell lines overexpressing HER2 and/or c-Src have been generated and will be tested for growth properties, heterocomplex formation and downstream signaling. Preliminary results indicate a HER2/c-Src complex in a cell line which overexpressed both kinases; no complex is seen in a cell line expressing c-Src alone. Cell lines generated will be tested for tumorigenicity in assays of [^{3}H]-thymidine incorporation, soft agar growth, and tumor formation in nude mice.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer tumor progression, HER2, c-Src, tyrosine kinase	26
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Allison P. Bebcock 7-14-97

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Results of previous studies by several laboratories have indicated that functional interaction between members of the HER receptor tyrosine kinase family, and c-Src, a non-receptor tyrosine kinase, may be involved in breast tumor formation. Overexpression and/or gene amplification of HER1 and HER2 is seen in approximately 20-30% of human breast tumors, and has been correlated with poor patient prognosis (Slamon *et al.*, 1987; Slamon *et al.*, 1989). Elevated $pp60^{c\text{-}Src}$ activity has been seen in a majority of breast tumors (Ottenholf-Kalff *et al.*, 1992), including tumors which express HER2 (Muthuswamy *et al.*, 1994). In addition, stable *in vitro* protein-protein interactions between the SH2 domain of c-Src and HER1 or HER2 have been noted in human breast tumor cell lines (Luttrell *et al.*, 1994). Taken together, this evidence suggests a potential role of HER1, HER2 and c-Src working together in human breast tumor formation and progression. Our laboratory has discovered an EGF-dependent physical association between another HER family member, the epidermal growth factor receptor (HER1), and c-Src in a murine fibroblast cell line engineered to overexpress both of these proteins. We have also demonstrated this association in five breast cancer cell lines known to overexpress the HER1 receptor (Biscardi *et al.*, manuscript submitted). In both the mouse model cell lines and human breast tumor cell lines, when HER1 and c-Src are found in complex with one another, there is a synergistic effect on tumorigenesis in vivo, as assayed by *de novo* DNA synthesis, growth in soft agar, and tumor formation in nude mice. These data imply both structural and functional interactions of c-Src and HER1, as well as a potentially critical step for tumor progression in breast cells.

Overexpression of HER2 is observed in a number of human cancers, including breast (Slamon *et al.*, 1989), ovarian (Berchuck, *et al.*, 1990), gastric (Kameda *et al.*, 1990), lung (Kern *et al.*, 1990) and prostate (Ware *et al.*, 1991). Activated HER2 can act as a potent transforming protein when overexpressed, both in breast epithelia cell lines (Pierce *et al.*, 1991) and in NIH3T3 cells (DiFiore *et al.*, 1987). Clearly, involvement of HER2 is implicated in the mechanism of many human neoplasias.

While it shares a high percentage of homology with the HER family of receptor tyrosine kinases (80% sequence homology between HER1 and HER2, Coussens *et al.*, 1985), no ligand has been identified for HER2. Initial studies indicated that the peptide heregulin, also known as *neu* differentiation factor (NDF), binds to the HER2 receptor, and causes increased phosphorylation of downstream substrates of HER2 (Holmes *et al.*, 1992; Wen *et al.*, 1991). However, it has since been demonstrated that heregulin is a ligand for family members HER3 and HER4. Upon binding one of these receptors, heregulin can cause homodimerization of HER3 or HER4, and heterodimerization of HER3/HER2 or HER4/HER2 (Carraway and Cantley, 1994; Carraway, III, *et al.*, 1995; Sliwkowski *et al.*, 1994; Plowman *et al.*, 1993). Dimerization and the subsequent trans-phosphorylation then stimulates cellular signaling pathways mediated through PI_3 kinase, and potentially other signaling molecules (Carraway *et al.*, 1995). Stimulation of cells with epidermal growth factor (EGF) is also believed to cause heterodimerization between HER1 and HER2, in addition to homodimerization of HER1 (Ullrich and Schlessinger, 1990). The heterodimerization properties of the HER family members is an area of intense study at this time and could be an important factor in the tumorigenic properties of these receptors.

The goals of this study are to determine whether interactions occur between c-Src and HER2 in a fashion similar to those which have been demonstrated between c-Src and HER1, and whether this interaction correlates with enhanced tumor formation. HER2 is very similar in structure to HER1, and phosphorylates similar downstream targets. Using C3H10T1/2 murine fibroblasts as a model system, we have engineered cell lines which overexpress HER2 and c-Src, either together or alone. A subset of these cell lines have been assayed for physical association of HER2 and c-Src, and remain to be assayed for changes in tumorigenic properties, changes in phosphorylation of the receptor, and changes in the receptor phosphorylation of downstream target molecules. Since interactions between HER2 and c-Src will first be assayed in a simplified model system, it will be possible to correlate significant tumorigenic changes with the overexpression of a distinct protein in an established cellular background. Comparisons of the tumorigenicity of wildtype and kinase-deficient HER2 in the model system may also allow for a direct test of cause and effect of the overexpression of HER2.

We have also assayed for similar changes in more complex systems, i.e. breast tumor tissue samples and a panel of breast cancer cell lines. These tissues and carcinoma cell lines have been screened for overexpression of HER2 or c-Src, *in vivo* association between HER2 and c-Src, and changes in tyrosine phosphorylation of potential downstream receptor target molecules. In addition, the carcinoma cell lines will be tested for their tumorigenic properties. In cases where a cell line overexpresses either HER2 or c-Src, then the missing partner will be overexpressed, and the cell line assayed for physical association of HER2 and c-Src, and any changes in phosphorylation of downstream targets and tumorigenic properties.

A model cell line overexpressing both HER1 and HER2, with or without c-Src, will also be constructed, in order to assay for influence of receptor heterodimerization on the synergistic effects on tumorigenicity. These studies will serve as a template for similar future investigations involving c-Src with HER3 and HER4. Performed both in model systems and in breast cancer cell lines, these investigations will identify cellular molecules active in breast cancer formation and progression, as well as potential molecules to target in breast cancer therapeutic strategies.

MATERIALS AND METHODS

Cell lines. Tumor cell lines MDA-MB-468, MDA-MB-231, MCF7 and ZR75-1 were obtained from N. Rosen (Sloan-Kettering, New York, USA). Tumor cell lines MDA-MB-175, UACC-893, UACC-812, SK-BR-3, MDA-MB-361, BT-474, BT-549, BT-20 and HS578Bst were obtained from American Type Culture Collection (location, USA). Tumor cells were maintained in Dulbecco's modified Eagle medium (DMEM) in 10% fetal calf serum. Where indicated, cells were stimulated with either purified mouse epidermal growth factor (EGF) (100ng/ml) or recombinant human heregulin-α (200ng/ml) (Sigma Chemical, St. Louis, MO, USA). The derivation and characterization of neomycin-resistant (Neo control) and c-Src overexpressor (5Hd47) C3H10T1/2 mouse fibroblasts have been described previously (Luttrell *et al.*, 1988; Wilson *et al.*, 1989; Maa *et al.*, 1995). C3H10T1/2 cell lines were maintained in DMEM with 10% fetal calf serum and 400μg/ml G418.

Overexpression of HER2 in C3H10T1/2 mouse fibroblasts. HER2 cDNA in a retroviral vector construct (LTR-1) (gift of P. DiFiore) (DiFiore *et al.*, 1987) were co-transfected along with pBABE puromycin-resistance vector (Morgenstern and Land, 1990) via Lipofectin reagent

(Life Technologies, Gaithersburg, MD, USA), into Neo (control) and 5Hd47 (c-Src overexpressors). Transfected colonies were selected with G418 (400 μg/ml) and puromycin (concentration). Mutants of HER2 transfected include activated, oncogenic HER2 (V659E), kinase-deficient HER2 (L753R), a potential auto-phosphorylation site mutant (Y877F), and a chimeric receptor containing the extracellular domain of HER1 (residues -24->+621 from the original sequence, Ullrich, *et al.*, 1984) with transmembrane and intracellular domains of the HER2 receptor (residues +654->+1255 from Coussens, *et al.*, 1985). Overexpression of HER2 and/or c-Src proteins was assayed by Western blotting, and fold increases over parental cell lines determined by scanning by a Molecular Dynamics densitometer (San Francisco, CA, USA).

Antibodies. C-Src antibodies used include mouse monoclonal antibodies (mAb) 2-17, directed against amino acids 2-17 (Quality Biotech, Camden, NJ, USA); mAbs GD11 and EB8, both directed against residues 92-128 in the SH3 domain (Parsons *et al.*, 1984; Parsons *et al.*, 1986); and mAb 327, which recognizes the SH3 domain (gift from J. Brugge). HER2-specific antibody (rabbit polyclonal), directed toward residues 1169-1186, and HER3-specific antibody, directed toward residues 1307-1323, were obtained from Santa Cruz Biotechnologies, Santa Cruz, CA, USA. Antibodies directed to the epidermal growth factor receptor (EGFR/HER1) mAbs 3A and 4A were provided by D. McCarley and R. Schatzman of Syntex Research, Palo Alto, CA, USA. Their epitopes map to amino acid residues 889-944 and 1052 and 1134, respectively (Maa, 1995). Anti-phosphotyrosine (p-Tyr) polyclonal antibody (RC20) was purchased from Transduction Laboratories (Lexington, KY, USA), and SHC polyclonal antibody was purchased from UBI (Lake Placid, NY, USA). Anti-phospho-MAPK antibody was purchased from Promega (Madison, WI, USA), and anti-pan MAPK antibody (B3B9) was the gift of M. Weber (Reuter *et al.*, 1995). Negative control antibodies include purified normal rabbit or mouse immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

Immunoprecipitation and Western immunoblotting. Cells were lysed in RIPA detergent buffer (10mM Tris-HCl pH 7.2, 1% Triton-X, 0.5% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM sodium orthovanadate, 50μg/ml leupeptin and 0.5% aprotinin). Tumor samples were minced with a scalpel and ground in a Dounce homogenizer in RIPA buffer. All lysates were cleared by centrifugation, and protein concentration determined by Bradford assay. For immunoprecipitation, 5 μg of antibody was added to 500 μg of cell lysate incubated while rocking at 4°C for 2 hours, and then incubated for an additional hour at 4°C with protein A-sepharose beads (Sigma Chemical). After incubation, samples were centrifuged, and the sepharose beads washed three times with RIPA buffer. The samples were then resuspended in 2X sample buffer (125mM Tris-HCl, 4% SDS, 10% glycerol, 0.02% bromophenol blue, 4% mercaptoethanol), run through a 7% SDS-polyacrylamide gel and immunoblotted according to previously published protocols (Luttrell *et al.*, 1988; Wilson *et al.*, 1989; Maa *et al.*, 1995). Binding of primary antibodies was visualized by ^{125}I-protein A used at 1 μCi/ml (New England Nuclear, USA), or by enhanced chemiluminescence (Amersham, Buckinghamshire, England).

RESULTS AND DISCUSSION

Development of stable HER2 overexpressing cells. The development and characterization of wildtype and mutant HER2-overexpressing cell lines in the C3H10T1/2 mouse fibroblast system has progressed, both in the presence and absence of the overexpression of c-Src. Four of ten

model cell lines have been obtained (Table I). Alternative transfection protocols are being discussed to develop the missing lines. We have examined the three cell lines which overexpress c-Src and kinase-deficient HER2 for heterocomplex formation, and in each of these cell lines, we have demonstrated that HER2 co-immunoprecipitates with c-Src using the monoclonal antibody 2-17 to c-Src.

Overexpression of c-Src and HER family members in human breast carcinoma cell lines. A panel of human breast carcinoma cell lines was analyzed by Western blotting for protein expression levels of c-Src and HER family members (Figure 1). Scanning densitometry of autoradiographs of the blots is shown in Table II. Nine of these cell lines exhibit expression of c-Src greater than two-fold above the normal control human breast epithelial cell line, HS578Bst. Many cell lines exhibit increased expression of HER1, HER2 or HER3, in various combinations. In the majority of cases, however, expression of HER1 and HER2 appears to be mutually exclusive in the same tumor cell line. This finding agrees with published literature which indicate that HER2 is typically expressed in earlier stage breast carcinomas (De Potter *et al.*, 1990; Gusterson *et al.*, 1988; Gusterson *et al.*, 1988b; Maguire *et al.*, 1992; Ramachandra *et al.*, 1990; van de Vijver *et al.*, 1988), while HER1 is expressed in later, more aggressive tumors (Battaglia *et al.*, 1988; Sainsbury *et al.*, 1987; Toi *et al.*, 1991).

Association of c-Src with HER2 in human breast tumor samples. A panel of human breast carcinoma tissue samples was analyzed by immunoprecipitation and Western blotting with antibodies to HER2 and c-Src for *in vivo* association between c-Src and HER2 (Figure 2). Three of thirteen tumors exhibit a specific c-Src/HER2 complex, compared to Neo cells. Tumors UVA156 and UVA263 were scored as positive for c-Src/HER2 complex formation, whereas MichN1 was scored as negative for complex formation. In addition, HER2/HER1 (UVA156 and UVA263) and HER2/HER3 (UVA156) complexes can be seen, indicating heterodimerization between HER family members in these tumors. Table III summarizes tumor type and grade, estrogen receptor (ER) status and lymph node involvement for each tumor, if known, as well as relative levels of c-Src and HER2 protein of each tumor. This complex formation between c-Src and HER2 has been demonstrated in transgenic mice mammary tumors (Muthuswamy *et al.*, 1994), but this is the first demonstration of an *in vivo* association in human breast carcinoma samples.

Physical association of c-Src with HER2 in human breast carcinoma cell lines. Similarly, a panel of human breast carcinoma cell lines were examined for a c-Src/HER2 complex upon EGF- and heregulin-stimulation. Four of the cell lines tested are represented in Figures 3 and 4 (MDA-MB-361, MDA-MB-453, MCF7, MDA-MB-468). Of the nine human breast tumor cell lines which overexpress both HER2 and c-Src, three exhibit constitutive association between c-Src and HER2 in serum-starved cells (MDA-MB-361, MDA-MB-453, UACC-812). The amount of HER2 which co-immunoprecipitates with the 2-17 α-c-Src antibody can be augmented when the cells are stimulated with 100ng/ml EGF for 5 minutes. However, heregulin stimulation (200ng/ml, 5 minutes) of the cells does not increase the amount of c-Src/HER2 complex above constitutive levels. These data suggest the association between HER2 and c-Src may be mediated through activation of HER1 and HER1/HER2 heterodimerization in these cell lines, and not through HER3 or HER4. In addition, the presence of constitutive complexes between c-Src and HER2 suggest that an growth factor autocrine loop may be at work in these cell lines.

Analysis of phosphotyrosine content of cellular proteins of human breast carcinoma cell lines. EGF and heregulin bind transmembrane receptors of the HER family expressed in breast carcinoma cells. Activation consequently leads to an activation of the receptor tyrosine kinase activity, and an increase in tyrosine phosphorylation of receptor substrates. To determine which substrates may be phosphorylated after EGF or heregulin treatment, and possibly be involved in downstream signaling cascades, we analyzed the phosphotyrosine content of cellular proteins by immunoblot with an α-phosphotyrosine specific antibody of whole cell lysates (Figure 5). A protein of approximately 185kDa is constitutively phosphorylated in two cell lines, MDA-MB-361 and MDA-MB-453, yet is not evident in MCF7 or MDA-MB-468 cells. The p-Tyr signal of this protein increases upon stimulation with heregulin. A protein of 170kDa is hyperphosphorylated in MDA-MB-468 cells upon EGF stimulation, as well as a spectrum of proteins between 43kDa and 69kDa. A similar spectrum of proteins increases in p-Tyr signal upon treatment with heregulin. In addition, proteins of approximately 97kDa to 140kDa exhibit an increase in p-Tyr signal upon heregulin treatment.

Signal transduction of SHC and MAPK in human breast carcinoma cell lines. In attempts to discern the signal transduction pathways which may be activated by EGF or heregulin when this complex is present, we have examined four of the breast carcinoma cell lines for phosphorylation of SHC and MAPK in the presence of EGF or heregulin (Figures 6 and 7). In one of the cell lines which overexpress HER2, and exhibits heterocomplex formation between HER2 and c-Src (MDA-MB-453), SHC is constitutively phosphorylated. This finding is consistent with the constitutive phosphorylation of MAPK in this cell line. However, MAPK phosphorylation is inducible by both EGF and heregulin, suggesting a pathway other than SHC may be contributing to the cumulative phosphorylation of MAPK, or perhaps different residues of MAPK become phosphorylated by EGF or heregulin stimulation. In another cell line which shows a heterocomplex between HER2 and c-Src, MDA-MB-361, where there is low constitutive phosphorylation of both SHC and MAPK, phosphorylation of both molecules is inducible by EGF. Taken together, these data suggest that there are multiple pathways to activate mitogenesis via MAPK in these cell lines, mediated by multiple HER family members.

Progress towards Specific Aims.

Specific Aim I. Derive stable cells lines overexpressing c-Src or wild-type HER2 alone or in combination and examine them for growth, morphology and tumorigenic properties. Other transformants to be assayed include activated HER2, kinase-mutant HER2, and a chimeric receptor containing the extracellular domain of HER1, and the transmembrane and intracellular domains of HER2.

Moderate progress has been achieved for this specific aim, and new methods of transfection are being attempted to generate the remaining stable HER2 clones.

Specific Aim II. Screen a panel of human breast carcinoma cell lines for overexpression of c-Src or HER2, or both. In cell lines overexpressing only one of these, overexpress the missing partner and assay for effects on tumorigenicity.

Screening of this panel is complete. Assays of tumorigenicity for the panel and transfections to overexpress the missing partner will be complete within one year.

Specific Aim III. Examine derived cell lines overexpressing c-Src and HER2 as well as breast carcinoma cell lines for *in vivo* association of c-Src with HER2. If *in vivo* association is evident, examine both c-Src and HER2 immunoprecipitated in the complex for novel sites of phosphorylation, and assay for differences in the phosphorylation state of downstream substrates of HER2 and c-Src.

Assays for c-Src/HER2 complex formation in breast carcinoma cell lines are complete, as well as assays for changes in phosphorylation of downstream substrates. Novel sites of phosphorylation for the HER2 receptor while in complex with c-Src will be completed within 9 months.

Specific Aim IV. Derive stable cell lines overexpressing wild-type HER1 and HER2 simultaneously, with and without overexpression of c-Src, and assay for changes in growth, morphology and tumorigenicity. Comparisons will be made between these cell lines and those derived to overexpress HER2 alone, with or without overexpressing c-Src.

This specific aim will be completed within the last year of funding.

CONCLUSIONS

1. HER family members as well as c-Src are overexpressed in the majority of human breast tumor cell lines and breast tumors tested (total of 27).

2. In 3 of 14 breast tumor cell lines and in 3 of 13 human breast tumors, HER2 and c-Src are physically associated in an immune complex. However, in contrast to the HER1/Src model system, overexpression of neither HER2 or c-Src is required for physical association. Similar to the HER1/c-Src model, the HER2/c-Src association in the cell lines is inducible by EGF but not by heregulin.

3. One of the 3 cell lines (MDA-MB-361) exhibiting the HER2/c-Src complex shows inducible SHC phosphorylation in response to both EGF and heregulin, while another (MDA-MB-453) shows constitutive phosphorylation of SHC, insensitivity to EGF, but inducibility to heregulin. These data indicate that there is no correlation between HER2/c-Src association and SHC phosphorylation.

4. MAPK shows low basal levels and activation in all cell lines (to varying degrees) in response to both EGF and heregulin. In the MDA-MB-453 cell lines, activation of MAPK in response to EGF is SHC-independent, while in MDA-MB-361 cells, activation of MAPK correlates with SHC tyrosine phosphorylation.

5. EGF-inducibility of HER2/c-Src association correlates with MAPK activation but not with SHC tyrosine phosphorylation.

REFERENCES

Battaglia, F., Scambia, G., Rossi, S., Panici, P.B., Bellantone B., Polizzi, G., Querzoli, P., Negrini, R., Iacobelli, S., Crucitti, F., and Mancuso, S. 1988. Epidermal growth factor receptor in human breast cancer: correlation with steroid hormone receptor and axillary lymph node involvement. Eur J Cancer Clin Oncol 24:1685-1690

Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J.T., Dodge, R., Clarke-Pearson, D.L., Marks, P., McKenzie, S., Yin, S. and Bast, R.C.J. 1990. Overexpression of HER2/*neu* is associated with poor survival in advanced ovarian cancer. Cancer Res 50:4087-4091

Biscardi, J.S., Belsches, A.P., and Parsons, S.J. 1997. Characterization of human epidermal growth factor receptor and c-Src in human breast tumor cells. Sumitted to Molecular Carcinogenesis.

Carraway, K.L., III, and Cantley L.C. 1994. A *neu* acquaintance for *erb*B3 and *erb*B4: a role for receptor heterodimerization in growth signaling. Cell 78:5-8

Carraway, K.L., III, Soltoff, S.P., Diamonti, A.J. and Cantley, L.C. 1995. Heregulin stimulates mitogenesis and phosphtidylinositol 3-kinase in mouse fibroblasts transfected with *erb*B2/*neu* and *erb*B3. J Biol Chem 270:7111-7116

Coussens, L., Yang-Feng, T.L., Liao, Y.C., Chen, E., Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U. and Ullrich, A. 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230:1132-1139

De Potter, C.R., Beghin, C., Makar, A.P., Vandekerckhove, D., and Roels, H.J. 1990. The *neu*- oncogene protein as a predictive factor for haematogenous metastases in breast cnacer patients. Int J Cancer 45:55-58

DiFiore, P.P., Pierce, J.H., Kraus, M.H., Segatto, O., King, C.R., Aaronson, S.A. 1987. *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237:178-182

Gusterson, B.A. Machin, L.G., Gullick, W.J., Gibbs, N.M., Powles, T.J., Elliott, C., Ashley, S., Monaghan, P. and Harrison, S. 1988. c-erbB-2 expression in benign and malignant breast disease. Br J Cancer 58:453-457

Gusterson, , B.A. Machin, L.G., Gullick, W.J., Gibbs, N.M., Powles, T.J., Price, P., McKinna, A., and Harrison, S. 1988b. Immunohistochemical distribution of c-erbB-2 in infiltrating and *in situ* breast cancer. Int J Cancer 42:842-845

Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D., Shepard, H.M., Kuang, W.-J., Wood, W.I., Goeddel, D.V. and Vandlen, R.L. 1992. Identification of heregulin, a specific activator of $p185^{erbB2}$. Science 256:1205-1210

Kameda, T., Yasui, W., Yoshida, K., Tsujino, T., Nakayama, H., Ito, M., Ito, H. and Tahara, E. 1990. Expression of *ERB*B2 in human gastric carcinomas: relationship between $p185^{ERBB2}$ expression and gene amplification. Cancer Res 50:8002-8009

Kern, J.A., Schwartz, D.A., Nordberg, J.E., Weiner, D.A., Greene, M.I., Torney, L. and Robinson, R.A. 1990. $p185^{neu}$ expression in human lung adenocarcinomas predict shortened survival. Cancer Res 50:5184-5187

Luttrell, D.K., Lee, A., Lansing, T.J., Crosby, R.M., Jung, K.D., Willard, D., Luther, M., Rodriguez, J., Berman, J. and Gilmer, T.M. 1994. Involvement of $pp60^{c\text{-}Src}$ with two major signaling pathways in human breast cancer. Proc Natl Acad Sci USA 91:83-87

Luttrell, D.K., Luttrell, L.M. and Parsons, S.J. 1988. Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblast that overexpress pp60$^{c\text{-}src}$. Mol Cell Biol 8:497-501

Maa, M.-C., Leu, T.-H., McCarley, D.J., Schatzman, R.C., and Parsons, S.J. 1995. Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. Proc Natl Acad Sci USA 92:6981-6985

Maguire, Jr., H.C., Hellman, M.E., Greene, M.I., and Yeh, I. 1992. Expression of c-erbB-2 in *in situ* and in adjacent invasive ductal adenocarcinomas of the female breast. Pathobiology 60:117-121

Morgenstern, J.P. and Land. H. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell lines. Nuc Acid Res 18:3587-3596

Muthuswamy, S.K. and Muller, W.J. 1995. Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor. Oncogene 11:271-279

Muthuswamy, S.K., Siegel, P.M., Dankort, D.L., Webster, M.A. and Muller, W.J. 1994. Mammary tumors expressing the *neu* proto-oncogene possess elevated c-Src tyrosine kinase activity. Mol Cell Biol 14:735-743

Ottenhoff-Kalff, A.E., Rijksen, G., van Beurden, E.A.C.M., Hennipman, A., Michels, A.A., and Staal, G.E.J. 1992. Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-*src* oncogene product. Cancer Res 52:4773-4778

Parsons, S.J., McCarley, D.J., Ely, C.M., Benjamin, D.C., and Parsons, J.T. 1984. Monoclonal antibodies to Rous sarcoma virus pp60^{Src} react with enzymatically active cellular pp60^{Src} of avian and mammalian origin. J Virology 51:272-282

Parsons, S.J., McCarley D.J., Raymond, V.W., and Parsons, J.T. 1986. Localization of conserved and nonconserved epitopes within the Rous sarcoma virus-encoded *src* protein. J Virology 59:755-758

Pierce, J.H., Arnstein, P., DiMarco, E., Artrip, J., Kraus, M.H., Lonardo, F., DiFiore, P.P., Aaronson, S.A. 1991. Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene 6:1189-1194

Plowman, G.D., Green, J.M., Culouscou, J.-M., Carlton, G.W., Rothwell, V.M., and Buckley, S. 1993. Heregulin induces tyrosine phosphorylation of HER4/p180^{erbB4}. Nature 366:473-475

Plowman, G.D., Culouscou, J.-M., Whitney, G.S., Green, J.M., Carlton, G.W., Foy, L., Neubauer, M.G. and Shoyab, M. 1993. Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc Natl Acad Sci USA 90:1746-1750

Ramachandra, S., Machin, L., Ashley, S., Monaghan, P. and Gusterson, B.A. 1990. Immunohistochemical distribution of c-erbB-2 in *in situ* breast carcinoma--a detailed morphological analysis. J Pathol 161:7-14

Reuter, C., Catling, A. and Weber, M.J. 1995. Immune complex kinase assays for mitogen-activated protein kinase and MEK. Methods Enzymol 255:245-256

Sainsbury, J.R.C., Farndon, J.R., Needham, G.K., Malcolm, A.J. and Harris, A.L. 1987. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cnacer. Lancet 1:1398-1402

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and McGuire W.L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177-182

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. and Press, M.F. 1989. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707-712

Sliwkowski, M.X., Schaefer, G., Akita, R.W., Lofgren, J.A., Fitzpatrick, V.D., Nuijens, A., Fendly, B.M., Cerione, R.A., Vandlen, R.L. and Carraway, K.L., III. 1994. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J Biol Chem 269:14661-14665

Toi, M., Osaki, A., Yamada, J., and Toge, T. 1991. Epidermal growth factor receptor expression as a prognostic indicator in breast cancer. Eur J Cancer 27:977-980

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee. J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M. and Seeburg, P.M. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309:418-425

Ullrich, A. and Schlessinger, J. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212

van de Vijver, M.J., Johannes, L.P. and Wolter, J.M. 1988. New Engl J Med 319:1239-1245

Ware, J.L., Maygarden, S.J., Koontz, W.W. and Strom, S.C. 1991. Immunohistochemical detection of c-erbB-2 protein in human benign and neoplastic prostate. Hum Pathol 22:254-258

Wen, D., Peles, E., Cupples, R., Suggs, S.V., Bacus, S.S., Luo, Y., Trail, G., Hu, S., Silbiger, S.M., Levy, R.B., Kosky, R.A., Lu, H.S. and Yarden, Y. 1992. *Neu* differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit.. Cell 69:559-572

Wilson, L.K., Luttrell, D.K., Parsons, J.T., and Parsons, S.J. 1989. $pp60^{c\text{-}Src}$ tyrosine kinase, myristylation and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-Src. Mol Cell Biol 9:1536-1544.

APPENDIX

Table I. Development of stable transfectant cell lines. Stable clones of C3H10T1/2 mouse fibroblasts which overexpress wildtype or mutant HER2 protein are listed, with the relative levels of expression above parental 10T1/2 cells shown in parentheses. C-Src overexpressors (5Hd47) cells express c-Src protein approximately 25-fold over endogenous 10T1/2 levels (Luttrell *et al.*, 1988, Wilson *et al.*, 1989).

Figure 1. Human breast carcinoma cell lines overexpress HER family members and c-Src protein. Lysates were prepared in RIPA detergent buffer and 100μg protein loaded per well on a 7% SDS-polyacrylamide gel, transferred onto nylon membrane and immunoblotted with the following antibodies: $p185^{HER2}$, Santa Cruz SC-284; $p180^{HER3}$, Santa Cruz SC-285; $p170^{HER1}$, 3A/4A; $p60^{c\text{-}Src}$, 2-17. Primary antibodies were visualized by ^{125}I-protein A.

Table II. Relative levels of HER family members and c-Src in human breast carcinoma cell lines. Autoradiographs from Figure 1 were analyzed by densitometry and compared to Hs578Bst, a normal human mammary epithelial cell line. The lowest detectable signal on each blot was set as 1.0, and fold difference of the cell lines determined. An * indicates a signal was below the limits of detection by Western blotting. Also included in the table are estrogen receptor (ER) status of the cell lines, as determined by Western blotting (Biscardi *et al.*, manuscript submitted), and physical association between c-Src and either HER1 or HER2, as determined by immunoprecipitation with monoclonal antibodies to c-Src.

Figure 2. C-Src and HER2 show *in* vivo association in human breast tumor samples. Tumor lysates were homogenized and lysed in RIPA detergent buffer, and immunoprecipitated with either α-Src (S), α-HER1 (H1), α-HER2 (H2), α-HER3 (H3) or mouse α–rabbit IgG (-) antibodies. IPs were then separated through a 7% SDS-polyacrylamide gel and transferred to nylon membrane. The membrane was cut between the 97kDa and 69kDa markers, and the top half probed with α-HER2 polyclonal antibody, and the bottom half probed with α-c-Src monoclonal antibody 2-17. Both primary antibodies were visualized by ^{125}I-protein A. (-) indicates mouse α-rabbit IgG as a negative control for immunoprecipitation.

Table III. Summary of Human Breast Tumors Tested for Physical Association of HER2 and c-Src. Human breast tumor samples obtained from the UVA Tissue Procurement Facility and the University of Michigan Tumor Bank were analyzed by immunoprecipitation and Western blotting for physical association between HER2 and c-Src. Tumor type and grade, ER and lymph node status, and relative levels of HER2 and c-Src protein are listed for each tumor.

Figure 3. EGF-inducible association between c-Src and HER2 in human breast carcinoma cell lines. Panel A: MDA-MB-361, MDA-MB-453, MCF7 and MDA-MB-468 cells were serum-starved, and stimulated with 100ng/ml EGF (+), or serum-free media (-). The cells were then lysed and immunoprecipitated with either α-Src (S), α-HER2 (H2) or mouse α–rabbit IgG (-) antibodies. IPs were then separated on a 7% SDS-polyacrylamide gel and transferred onto nylon membrane. The upper half of the membrane was probed with α-HER2 primary antibody,

and the lower half probed with α-Src (2-17) primary antibody. Both primary antibodies were visualized by ^{125}I-protein A. Panel B: Cell lysates from unstimulated MDA-MB-361 cells were immunoprecipitated with four antibodies to c-Src, 2-17, GD11, mAb327, and EB8, and four negative control antibodies, rabbit α-mouse IgG, mouse α–rabbit IgG, rabbit α–goat IgG and purified mouse IgG. The IPs were immunoblotted as above.

Figure 4. Heregulin-independent association between c-Src and HER2 in human breast carcinoma cell lines. MDA-MB-361, MDA-MB-453, MCF7 and MDA-MB-468 cells were serum-starved, and stimulated with 200ng/ml heregulin (+), or serum-free media (-). The cells were then lysed and immunoprecipitated with either α-Src (S), α-HER2 (H2) or mouse α–rabbit IgG (-) antibodies. IPs were then separated on a 7% SDS-polyacrylamide gel and transferred onto nylon membrane. The upper half of the membrane was probed with α-HER2 primary antibody, and the lower half probed with α-Src (2-17) primary antibody. Both primary antibodies were visualized by ^{125}I-protein A.

Figure 5. EGF- and heregulin-inducible tyrosine phosphorylation of cellular proteins of human breast carcinoma cell lines. Cell cultures were serum-starved overnight, and stimulated for five minutes with either EGF (100ng/ml) (Panel A) or heregulin-α (200ng/ml) (Panel B). Lysates were prepared in RIPA detergent buffer and 100μg protein loaded per well on a 7% SDS-polyacrylamide gel, transferred onto nylon membrane and immunoblotted with α-phosphotyrosine (p-Tyr) antibody. The primary antibody was recognized by enhanced chemiluminescence (ECL).

Figure 6. Constitutive, EGF- and heregulin-inducible tyrosine phosphorylation of SHC in human breast carcinoma cell lines. Cell cultures were serum-starved overnight, and stimulated for five minutes with either EGF (100ng/ml) (Panel A) or heregulin-α (200ng/ml) (Panel B). Lysates were prepared in RIPA detergent buffer, and immunoprecipitated with α-SHC antibody. IPs were separated on a 7% SDS-polyacrylamide gel, transferred onto nylon membrane and immunoblotted with α-phosphotyrosine (p-Tyr) antibody or α-SHC antibody. The primary antibody was recognized by ^{125}I-protein A.

Figure 7. Activation of MAP kinase by EGF and heregulin-α in human breast carcinoma cell lines. Cell cultures were serum-starved overnight, and stimulated for five minutes with either EGF (100ng/ml) (E) or heregulin-α (200ng/ml) (H). Lysates were prepared in RIPA detergent buffer and 100μg protein loaded per well on a 7% SDS-polyacrylamide gel, transferred onto nylon membrane and immunoblotted with either α-phospho-MAPK antibody or α-MAPK (B3B9). The primary antibody was recognized by ^{125}I-protein A.

Table I.
Development of stable transfectant cell lines.

Constructs Needed:	Control Background:	c-Src Overexpressor:
wildtype HER2	✓ 3 (3.0)[1]	
oncogenic HER2 (V659E)	✓ 4 (5.1)	
HER1/HER2 chimera		
kinase-deficient HER2 (L753R)		✓ 3 (24.6)
phosphorylation-site mutant HER2 (Y877F)	✓ 3 (6.3)	

[1]Indicates number of clones obtained and (fold overexpression).

Human Breast Carcinoma Cell Lines Overexpress HER Family Members and c-Src Protein

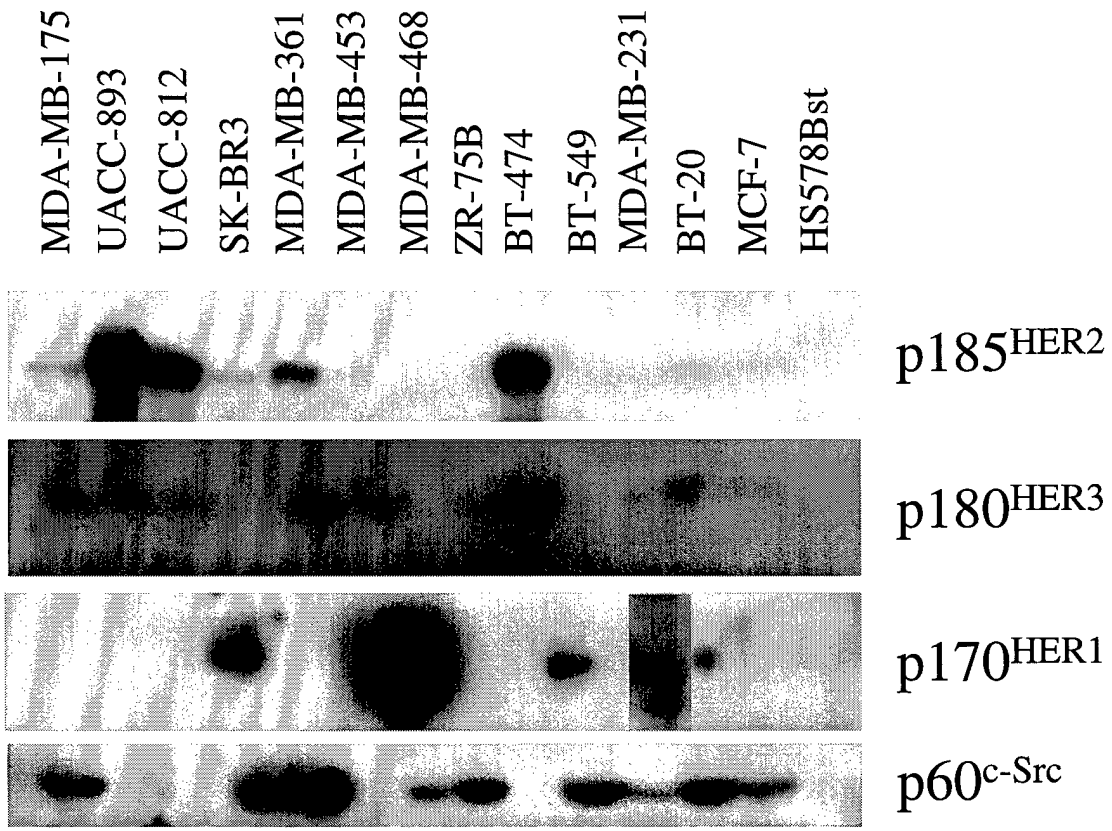


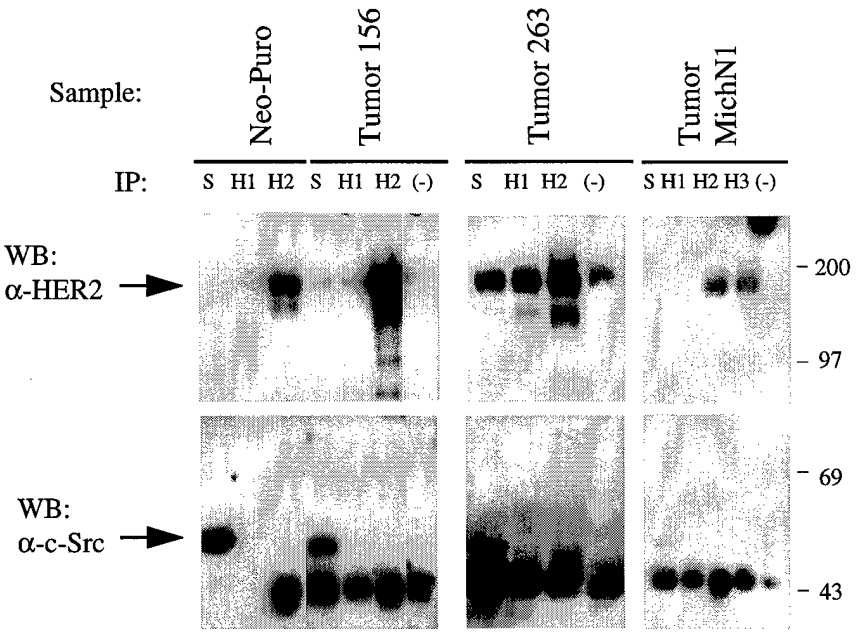
Figure 1

Table II
Human Breast Carcinoma Cell Lines
Relative levels of HER family members and c-Src

						Association of c-Src with:	
cell line	HER1	HER2	HER3	c-Src	ER	HER1	HER2
MDA-MB175	*	2.2	6.1	9.9	+	-	-
UACC-893	*	72.8	6.7	*	-		
UACC-812	*	50.8	6.1	*	-	-	+
SK-BR-3	12.6	2.5	*	19.4	-	+	-
MDA-MB361	*	12.8	10.9	37.4	+	-	+
MDA-MB453	*	1.5	6.1	*	-	-	+
MDA-MB468	39.5	*	*	4.9	-	+	-
ZR75-B	*	*	1.1	6.2	+	-	-
BT-474	*	61.8	26.4	*	+	-	
BT-549	6.9	*	*	13.4	-	+	-
MDA-MB231	7.6	1.1	1.0	2.9	-	+	-
BT-20	3.4	1.9	5	13.5	-	+	-
MCF-7	1	1	2.3	6.4	+	-	-
Hs578Bst[1]	1	*	*	1	+	-	-

[1]normal human breast epithelial cells used as negative control
(*) = below limits of detection
no entry in table indicates no data

c-Src and HER2 Associate in Breast Tumor Samples



Relative Levels of HER2 and c-Src

Sample	Neo-Puro	T156	T263	Mich N1
HER2	+	+++	+++++	+++
Src	+++	+++	+++	-

Figure 2

Table III

Summary of Human Breast Tumors Tested for Physical Association of HER2 and c-Src

Tumor	Tumor Type	ER	Lymph Node	Src/HER2 Association	c-Src	HER2
UVA103	IVDC[1], grade 3/3	-	-	-	ND[6]	ND
UVA156	IDC[2], grade 2/3	-	+	+	+++	+++
UVA226	IDA[3], grade 2/3	+		+	++	+++
UVA263	IDC, grade 3/3		+	+	+++	+++++
UVA387	ILC[4]	+		-	+	*[7]
UVA399	IDC, grade 3/3	-		-	+	+++
UVA454	IDC, grade 3/3	-	+	-	++	++
UVA616	IVDC, grade 3/3	-	+	-	ND	ND
MICHN1	medullary, grade 3/3			-	*	+
MICHP1	#[5]			-	*	++
MICHP2	#			-	*	+
MICHP3	#			-	*	+
MICHP4	#			-	*	+

n = 13

3/13 = 23%

[1]IVDC = invasive ductal carcinoma

[2]IDC = infiltrating ductal carcinoma

[3]IDA = infiltrating ductal adenocarcinoma

[4]ILC = infiltrating lobular carcinoma

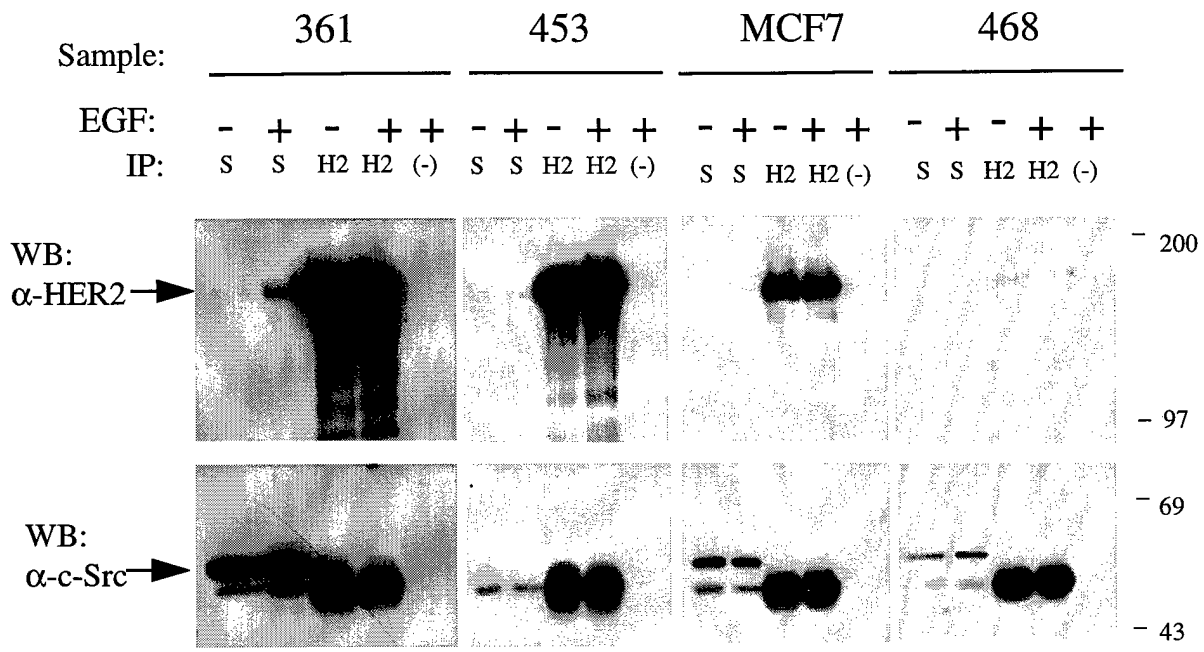
No entry in table = no data

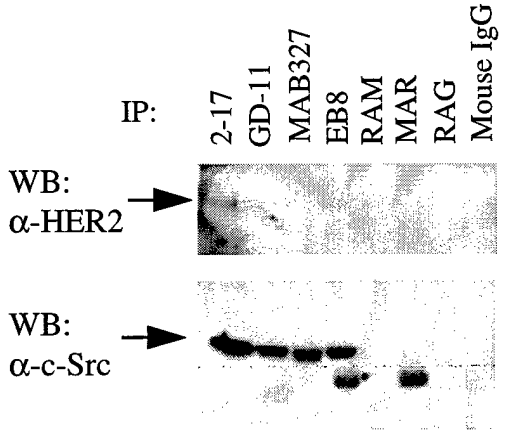
[5]# = data currently not available

[6]ND = not done

[7]* = below limits of detection

EGF-inducible Association between c-Src and HER2 in Human Breast Cancer Cell Lines





IP of c-Src from 361 lysates with different α-c-Src antibodies

Figure 3
22

Heregulin-independent Association between c-Src and HER2 in Human Breast Cancer Cell Lines

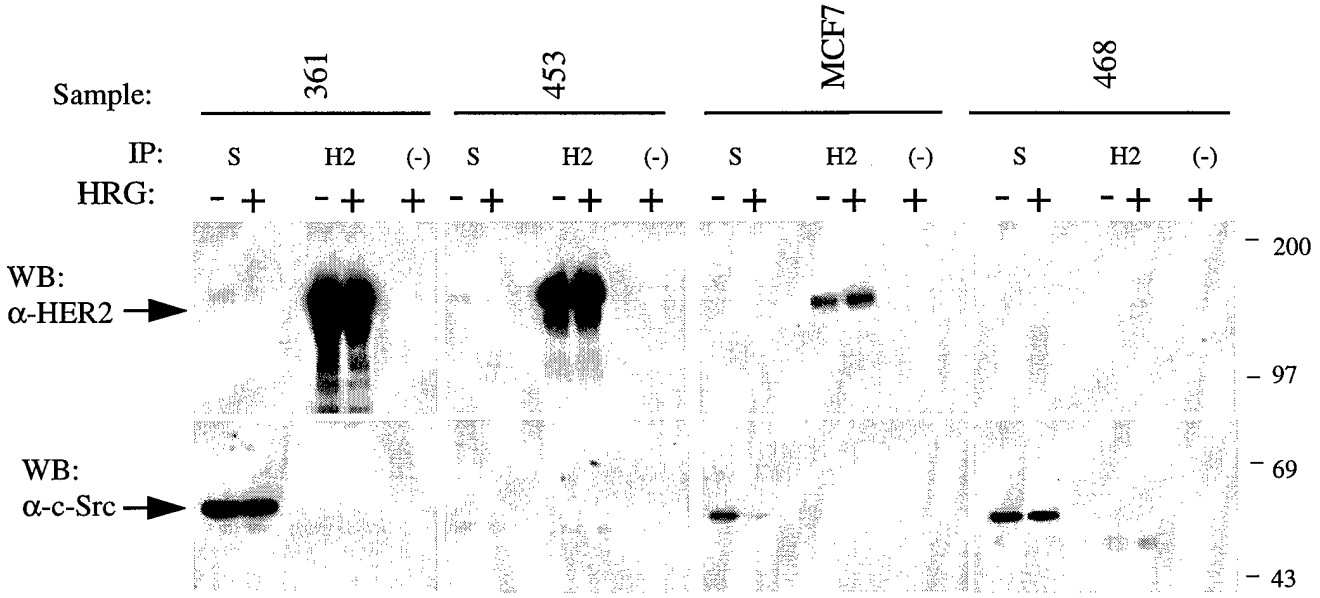
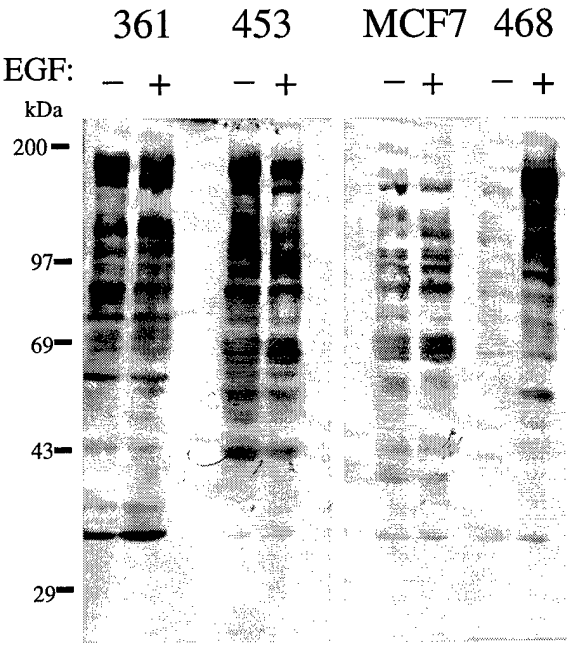


Figure 4
23

EGF- and Heregulin-inducible Tyrosine Phosphorylation of Cellular Proteins of Human Breast Carcinoma Cell Lines

A.



B.

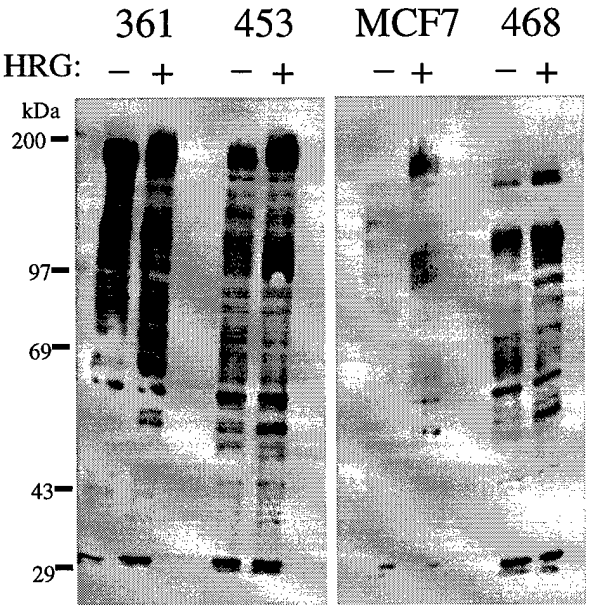


Figure 5

Constitutive, EGF- and HRG-inducible Tyrosine Phosphorylation of SHC in Human Breast Cancer Cell Lines

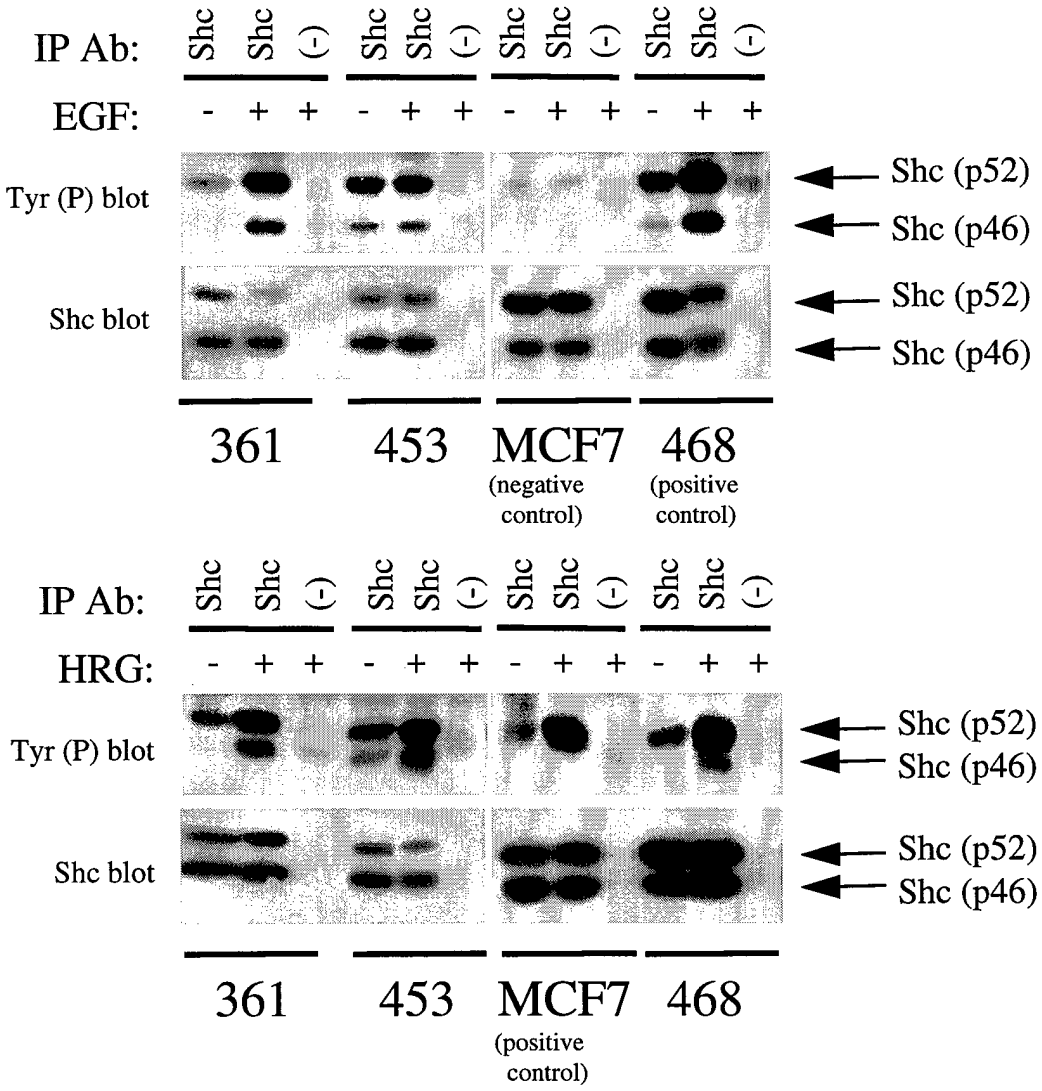


Figure 6

Activation of MAP Kinase by EGF and Heregulin in Breast Tumor Cell Lines

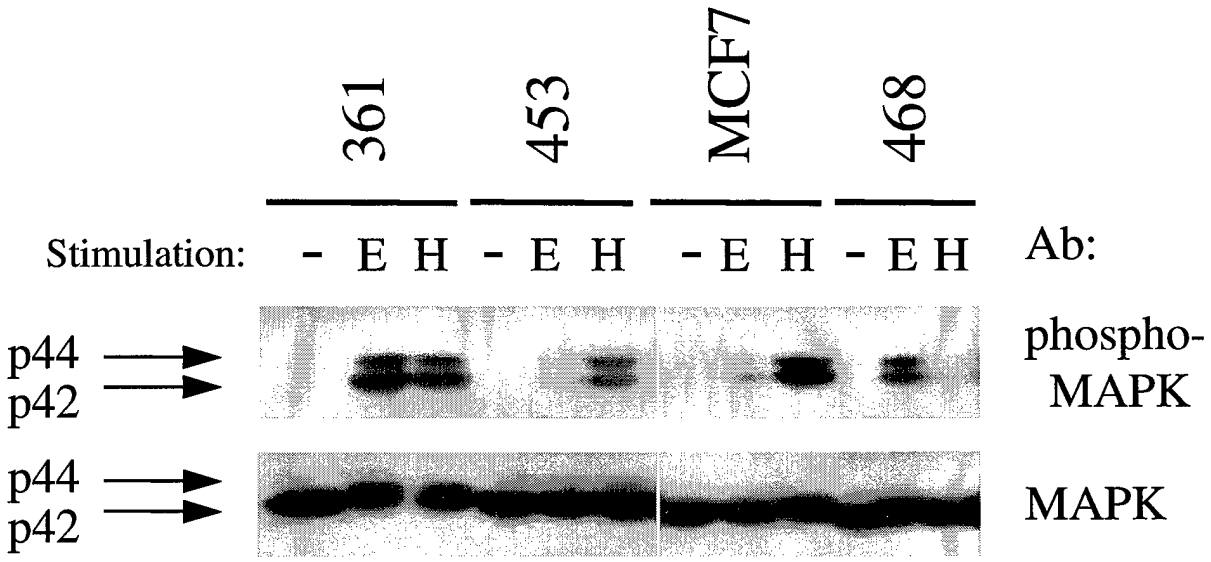


Figure 7
26